



**US Army Corps
of Engineers®**
Engineer Research and
Development Center

Community Composition of Bacterial Biofilms Formed on Simple Soil Based Bioelectro- chemical Cell Anodes and Cathodes

David B. Ringelberg, Karen L. Foley,
and Charles M. Reynolds

April 2012

Community Composition of Bacterial Biofilms Formed on Simple Soil Based Bioelectro- chemical Cell Anodes and Cathodes

David B. Ringelberg, Karen L. Foley, and Charles M. Reynolds

*Cold Regions Research and Engineering Laboratory
U.S. Army Engineer Research and Development Center
72 Lyme Road
Hanover, NH 03755*

Final report

Approved for public release; distribution is unlimited.

Prepared for U.S. Army Corps of Engineers
Washington, DC 20314-1000

Under Installation Technology Program

Abstract:

Microbial fuel cells (MFC), as bioelectrochemical systems, hold promise as a sustainable source of energy for use in novel environments and settings. Although electrode biofilms, both anode and cathode, are critical to the production of power in these systems, the taxonomies of the biofilms that form are not fully understood. The specific objectives of the current study were to classify the bacteria that enriched onto fuel cell electrodes, with three biogeochemically distinct surface soils serving as the inocula. Following 1000 hours of incubation under saturated soil conditions, the community composition of the anode and cathode bacterial biofilms was quantified by culture, fatty acid profile (FA), and terminal restriction fragment length polymorphisms (TRFLP). The three soils produced open circuit voltages of between 60 and 120 mV, but only one soil maintained voltage under resistance (60 mV at 10,000 Ω). Fatty acid profiling identified differences among anode, cathode, and bulk soil microbial communities, specifically a greater relative abundance of terminally branched saturated FA in the bulk soils and a greater relative abundance of monounsaturated and cyclopropane FA on electrode surfaces. Bacteria cultured from both the anode and cathode biofilms included species of Actinobacteria; however, only the anode biofilms produced species of Firmicutes and Proteobacteria. Analysis of TRFLP profiles putatively identified a diversity of bacteria in the biofilms recovered from both electrode surfaces. The most predominant organisms detected were the α - and β -Proteobacteria, specifically the Rhizobiales, Rhodobacterales, and Burkholderiales. Although the detection of similar community fingerprints on both anodes and cathodes was not anticipated, the diversity of organisms putatively identified indicated a complexity that would support a coupled nitrogen cycle, one capable of facilitating the transfer of electrons to the soil cell anodes and from the soil cell cathodes.

DISCLAIMER: The contents of this report are not to be used for advertising, publication, or promotional purposes. Citation of trade names does not constitute an official endorsement or approval of the use of such commercial products. All product names and trademarks cited are the property of their respective owners. The findings of this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

DESTROY THIS REPORT WHEN NO LONGER NEEDED. DO NOT RETURN IT TO THE ORIGINATOR.

Table of Contents

Abstract.....	ii
List of Figures and Tables	iv
Preface	v
1 Introduction.....	1
2 Materials and Methods.....	3
2.1 Soil characteristics	3
2.2 Bioelectrochemical Cells	4
2.3 Sample collection	4
2.4 Sample analysis.....	5
2.4.1 <i>Culturable bacteria</i>	5
2.4.2 <i>Lipid assay</i>	5
2.4.3 <i>Terminal restriction fragment length polymorphisms (TRFLP)</i>	6
2.4.4 <i>Statistical analysis</i>	7
3 Results and Discussion.....	8
3.1 Anode Biofilms.....	10
3.2 Cathode Biofilms	19
4 Summary and Conclusion.....	22
References.....	23
Report Documentation Page	

List of Figures and Tables

Figures

Figure 1. Actual soil cells with associated schematics.....	4
Figure 2. Voltage, open circuit potential, measured from the three soil cells over a 100 hour period.....	9
Figure 3. 2D plot illustrating results from the extraction of the 1 st two principal components from a dataset comprised of arcsine square root transformed fatty acid molar percentages..	12
Figure 4. A means comparison of fatty acid methyl ester (FAME) structural class molar percentages (mol%) across the averaged soil profiles (top, middle and bottom, n=9), anode attached soils and biofilms (n=5), cathode biofilms (n=3), and catholyte (n=2).....	13
Figure 5. Recovered and retained terminal restriction fragments (TRF) for each of the three soil cells (FG, FW, and CRL) following three restriction digests (Hha I, Msp I, and Rsa I) of bacterial 16S rRNA amplicons of electrode (anode and cathode) biofilm DNA.....	14
Figure 6. Pie charts of each of the three soil cell anode and cathode biofilm terminal restriction fragments that could be putatively identified through an in silico digest of the ribosomal database.	16

Tables

Table 1. Geochemical characteristics are provided for each soil used to fill the anodic chamber of the three test fuel cells.....	3
Table 2. Relative molar percentages and absolute abundances of prokaryotic and eukaryotic fatty acid (FA) biomarkers detected in the soils, top middle and bottom fractions, on the electrodes, biofilm and attached soil, and in the catholyte of the three test cells are provided.	11
Table 3. Factor variable correlations (loadings) between prokaryotic fatty acid (FA) profiles and the 1 st principal component that resulted following the principal components analysis of soil, electrode, and catholyte arcsine square root transformed fatty acid molar percentages.	13
Table 4. Putative identifications for retained terminal restriction fragments of amplified 16S rRNA DNA recovered from the anodes and cathodes of the three test cells following the in silico digest of the ribosomal database.....	17
Table 5. Enriched, isolated, and identified bacteria recovered from the anode and cathode biofilms of each of the three test cells.	19

Preface

The work was performed by David B. Ringelberg, Karen L. Foley, and Dr. Charles M. Reynolds (Biogeochemical Sciences Branch, Terrence M Sobecki, Chief), U.S. Army Engineer Research and Development Center–Cold Regions Research and Engineering Laboratory (ERDC-CRREL). At the time of publication, Dr. Justin Berman was Chief of the Research and Engineering Division. The Deputy Director of ERDC-CRREL was Dr. Lance Hansen, and the Director was Dr. Robert Davis.

The use of trade, product, or firm names in this report is for descriptive purposes only and does not imply endorsement by the U.S. Government. The tests described and the resulting data presented herein, unless otherwise noted, were obtained from research conducted under the Installation Technology Program of the U.S. Army Corps of Engineers by the USAERDC. Permission was granted by the Chief of Engineers to publish this information. The findings of this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

COL Kevin J. Wilson was the Commander and Executive Director of ERDC, and Dr. Jeffery P. Holland was the Director.

1 Introduction

In 1912, Michael Potter identified an electrical effect that accompanied the microbial decomposition of organic matter. Although the ability of bacteria to catalyze the conversion of organic matter into electricity has been known for over 75 years, the use of bacteria in fuel cells to generate appreciable electrical power is a more recent occurrence. In 2003, Chaudhuri and Lovely were able to show that a novel bacterium, *Rhodospirillum rubrum*, could transfer electrons directly to a graphite electrode while oxidizing a carbon substrate. The organism derived energy for growth and produced electricity from the coupled process. Rabaey et al. (2004) then demonstrated that microbial fuel cell (MFC) anodes can enrich for anodophilic communities that form biofilms capable of self-mediating electron transfer, resulting in stable and long term power densities. Soluble redox mediators or electron shuttles have been identified that facilitate electron transfer to fuel cell anodes (Rabaey et al. 2005) and, as it turns out, the electron shuttles produced by one bacterium can be utilized by other species of anodic bacteria (Pham et al. 2008). Certain bacteria have also been shown to produce conductive pilus-like structures, nanowires that enable a direct transfer of electrons from cell membrane to the anode surface (Reguera et al. 2005). That bacteria are capable of colonizing MFC electrodes and that they possess the molecular traits to enable electron transfer to these electrodes has been established. Research is now focusing on the breadth of anodophilic bacteria and microbes that exist in nature and into the factors that regulate electron transfer mechanisms.

The large percentage of studies examining bioelectrochemical system (BES) performance have focused on sewage sludge and wastewater as bacterial inocula, where anaerobic growth within the anodic chamber produces the electrons that when reduced at the cathode result in lower wastewater CODs (Alterman et al. 2006). Recent studies have begun looking into the nature of these biofilms in terms of community taxonomy and the activity related to electron transfer and power production. A number of studies have identified the Proteobacteria, including members of the alpha, beta, gamma, and delta classes, as being prominent anodophilic organisms (Kim et al. 2004; Phung et al. 2004; Kim et al. 2007; Chung and Okabe 2009). Molecular analyses, specifically of the 16S rRNA eubacterial gene, have also identified the presence of Firmicutes and Actinobacteria

on wastewater enriched anodes (Chung and Okabe 2009; Phung et al. 2004, respectively).

Of growing interest is the performance of MFC under aerobic conditions. Traditional BES used a two-chamber system where bacteria in an anode compartment were separated from the cathode compartment by a polymeric proton exchange membrane (PEM). Many of these MFC used aqueous cathodes with oxygen supplied via air sparging. Advances in design demonstrated that BES employing air-cathodes and the absence of a PEM were still efficient and effective at generating electricity (Liu and Logan 2004). In addition, these forms of BES were less costly and amenable to aerobic applications, such as in riverine systems. Acknowledgement of military as well as commercial uses of MFC for continued surveillance systems or for powering environmental sensors has lead to developments in the miniaturization of BES. Novel designs have been used to minimize anode cathode distances and to take advantage of 3D electrode materials to significantly increase electrode surface areas (Shantaram et al. 2005; Ringeisen et al. 2006).

Although the interaction between an anode and a microbial biofilm is a critical component of any bioelectrochemical system, microbe facilitated electron transfer off of fuel cell cathodes can also play an integral role. The reduction of O₂ at the cathode is often a significant bottleneck in BES efficiency and chemical catalysts, such as platinum, pyrolyzed iron, pthalocyanine, and cobalt tetramethylphenylporphrin, have all been used successfully to facilitate O₂ reduction. However, Clauwaert et al. (2007) showed that bacteria can also facilitate the O₂ reduction on the cathode surface and the formation of a cathode microbial biofilm, i.e., a biocathode, has appeal in terms of BES performance in terms of cost savings and improved power sustainability.

Microbial fuel cells as bioelectrochemical systems hold promise as a sustainable source of energy for use in novel ways and in novel environments. Metagenomics studies of anodic biofilms have and will likely continue to shed new light on the breadth of community functional dynamics that BES systems are capable of supporting (Liu et al. 2010). In this study we describe the molecular trait of three different soil based bioelectrochemical systems. We provide a polyphasic taxonomic description of the anodic and cathodic bacterial biofilms and identify common characteristics for use as a base for further development and refinement of BES for use in terrestrial environments.

2 Materials and Methods

2.1 Soil characteristics

Characteristics of the three soils examined in the study are provided in Table 1. Two of the study soils, abbreviated FG and FW, were collected from areas outside of Anchorage, AK, and the third soil, abbreviated CRL, was collected from the Cold Regions Research and Engineering Laboratory, Hanover, NH. The soils were all air dried, sieved to 4.75 mm (FG and FW soils) and 2.00 mm (CRL soil) and stored at room temperature. Prior to introduction into the bioelectrochemical cells, each soil was brought to 0.33 bar moisture content and incubated at room temperature for 72 hours.

Table 1. Geochemical characteristics are provided for each soil used to fill the anodic chamber of the three test fuel cells.

		Fairbanks, AK USA	Fairbanks, AK USA	Hanover, NH USA
		FW	FG	CRL
pH		7.8	5.6	6.5
soluble salts	mmhos/cm	0.10	0.13	0.18
CEC	meq/100g	16.6	2.3	2.7
TOC/O.M.	%	1.4	11.1	2.5
NO ₃ -N	ppm	6.0	7.8	2.4
NH ₄ -N	ppm	6.3	<3.9	5.1
N/C ratio	NH ₄ /TOC	4.6	0.4	2.0
P	ppm	4.4	0.8	2.2
K	ppm	39	16	57
Ca	ppm	1345	294	533
Mg	ppm	97	97	40
Fe	ppm	nd	3.5	0.6
Mn	ppm	nd	8.1	1.2
Cu	ppm	nd	0.0	0.0
Zn	ppm	3.8	0.2	1.2
Texture		sandy loam	silty loam	sandy loam
sand	%	49	29	49
silt	%	46	60	44
clay	%	5	12	7
nd = not detected				

2.2 Bioelectrochemical cells

A schematic of the bioelectrochemical cells is provided in Figure 1. Each cell consisted of an anodic chamber filled with soil and separated from the surrounding cathodic chamber by a cation exchange membrane (CMI-7000S, Membranes International, Inc.). The cation exchange membrane was sealed to the bottom of the glass jar with paraffin wax (~2 cm height). Each chamber, anode and cathode, contained a graphite electrode of equal dimensions, 3 mm deep \times 5 mm wide \times 100 mm long. The anodic chamber was loaded with ~5 g of soil, tamped, the electrode placed in the center, another 5 g of soil added and tamped around the electrode, etc., until the chamber was filled to within 1–2 mm of the top. The cathodic chamber was filled with deionized sterile water and continuously sparged with air. The electrode was then suspended in the water between the glass jar and the CEM. A small portion of the anodic soil, ~ 0.1 g, was added to the cathodic water as inocula. Voltage was measured across the two electrodes every hour using a digital multimeter (Kiethly Instruments) either as an open voltage or across a 10,000 Ω resistor.

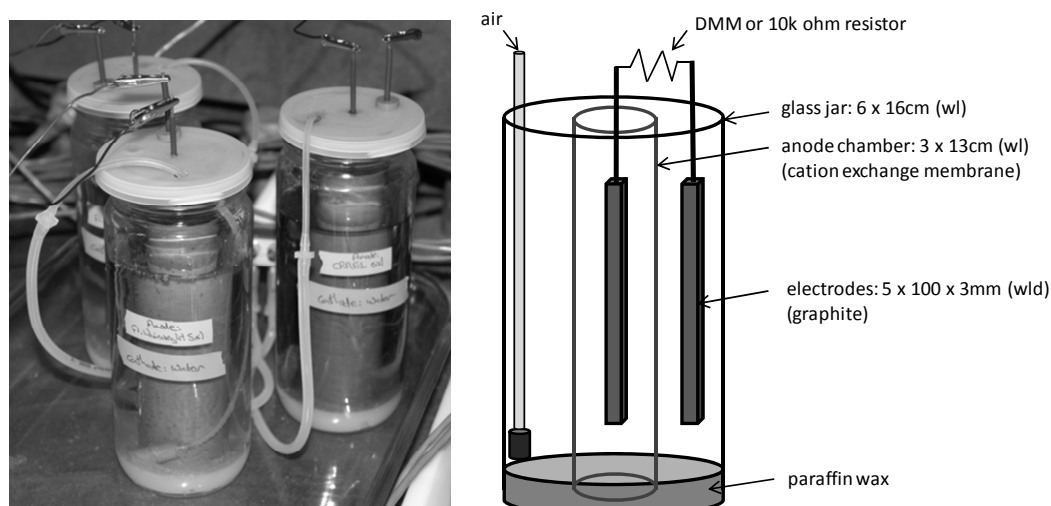


Figure 1. Actual soil cells with associated schematics.

2.3 Sample collection

Each BES cell was sampled to obtain taxonomic descriptions of the electrode biofilms and the surrounding media, i.e., soil or water. Although in some instances, sufficient material was not obtained for a particular assay, the sampling scheme outlined below was followed for each cell. Anode electrodes were first pulled from the soil columns and tapped gently to remove loosely attached soil clumps. The electrodes were then dipped in

sterile milli-Q water to recover the attached soils (AS), which were then collected by centrifugation at 8400 rpm for 20 minutes. The supernatant was decanted and the soils resuspended in 2 mL of sterile water for subsequent assay of lipids (0.8 mL), nucleic acids (0.8 mL), and culturable cells (0.4 mL). Biofilms remaining on electrodes were then recovered by swabbing each side with separate 5- × 5-mm sections of autoclaved (15 min. at 5.7 kPa) glass fiber filter paper (GF/A Whatman). One third of each swab was removed, suspended in 2 mL of sterile water and vortexed vigorously before removing 0.2-mL aliquots for cell culture. The remaining swabs were then directly assayed for either lipids or nucleic acids. Anode soils were separated into three sections, top, middle, and bottom, and mixed using a spatula before aliquots were removed to assay for lipids (2 g) and nucleic acids (0.2 g). Both the catholyte solution and the cathode electrodes were then sampled. Cells were recovered from the catholyte by centrifuging 30 mL at 9400 rpm for 20 minutes. The cell pellets were then resuspended in 1.6 mL of sterile milli-Q water and 0.8 mL was removed for determining lipid content and 0.8 mL for determining nucleic acid content. Cathode electrodes were processed in the same manner as the anode electrodes, excluding the step of dipping the electrode in sterile milli-Q water. Culture for bacterial isolation was not performed on either the catholyte solution or the anode soils.

2.4 Sample analysis

2.4.1 Culturable bacteria

Serial dilutions of each 0.2–0.4 mL aliquot was spread plated onto PTYG agar [5 g Peptone, 5 g tryptone, 5 g yeast extract, 10 g glucose, 0.6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per 1 L water at pH 7.0] and R2A agar (Difco, Becton Dickinson). Following 48 hours of incubation at 25°C, single colonies were picked and re-streaked for isolation. Isolated colonies were then identified by fatty acid profiles using the Microbial Identification System (MIDI Inc.) using Sherlock v4.0 software.

2.4.2 Lipid assay

In situ fatty acid profiles were obtained from the bulk anode soils, the cathode waters, and the anode and cathode electrode swabs. Briefly, total lipids were extracted from sample materials in a volume of chloroform:methanol:phosphate buffer (50 mM PO_4 at pH 7.4) in the ratios of 1:2:0.8 (v:v:v) (White and Ringelberg 1998). Soils, 2 g, and filter papers

were extracted in 3.8 mL of the extractant. For the catholyte and loosely attached soils, 0.8 mL of the suspensions was used in place of the 50 mM PO_4 buffer. Following phase separation and recovery of total lipids, a strong acid methanolysis was used to form fatty acid methyl esters (FAME), which were then recovered in a volume of hexane:chloroform (4:1, v:v). The FAME were then further separated, quantified, and identified by gas chromatography/mass spectrometry. Methyl nonadecanoate, 50 pmol μL^{-1} was used as an internal standard and individual fatty acids were expressed on a mole per gram basis. The relative molar percentage of each fatty acid was used for statistical comparisons.

2.4.3 Terminal restriction fragment length polymorphisms (TRFLP)

Total nucleic acids were recovered from the anode filter paper swabs using the PowerPlant™ DNA Isolation Kit (Mo Bio Laboratories). A Precellys 24 homogenizer (Bertin Technologies) was used to facilitate cell lysis. The primer pair 27F-FAM (5' – FAM- aga gtt tga tcc tgg ctc ag – 3') and 926R (5' – ccg tca att cct ttr agt tt – 3') was used to amplify a 16S rDNA fragment of approximately 915 bp in length. Approximately 100–200 ng of the amplicon was then purified on a QiaQuick PCR purification spin column (Qiagen) before digestion with 5 U of the restriction enzymes HhaI, MspI and RsaI at 37°C for 3 hours. Enzyme activity was stopped by heating at 60°C for 30 minutes. The digested amplicons were then desalted on Qiaquick nucleotide removal spin columns (Qiagen) and the concentrations adjusted to approximately 100 ng μL^{-1} . Purified fragments were then separated by capillary electrophoresis (15-s injection time at 15 kV, 15 kV electrophoretic voltage for 45 min.) on an Applied Biosystem 310 genetic analyzer. Fragments were sized against an x-rhodamine labeled 1000 bp ladder (MapMarker 1000, Bioventures, Inc.) using the local southern method within Peak Scanner software (v1, Applied Biosystems). Resulting TRF profiles were post-processed following the outline of Dunbar et al. (2000). TRF area percentages were then imported into the MICA 3 T-RFLP analysis (PAT+) program to perform in silico digests of the ribosomal database (release 10 update 12) of good quality 16S rRNA fragments (Shyu et al. 2007). Identifications resulting from each digest were compared using the on-line software program VENNY (Oliveros 2007) to produce putative identifications at the phylum, class, and order levels.

2.4.4 Statistical analysis

Principal components analysis was performed on arcsine square root transformed fatty acid molar percentages (Statistica v8). Means comparisons was by Tukey honest significant difference via a post hoc analysis and significance assumed at a $p < 0.05$.

3 Results and Discussion

The primary purpose of the simple soil cells was to facilitate anodic colonization by the extant soil bacteria. To date, there have been few efforts investigating soils as potential anode inocula (Schamphelaire et al. 2008; Schamphelaire et al. 2010; Ishii et al. 2008a,b; Jiang et al. 2010). The intent of this study was to augment these investigations by providing a polyphasic description of biofilms enriched onto graphite electrodes. Soils are non-ideal environments for MFC operation primarily because of the presence of the preferred electron acceptor oxygen and to existing constraints to mass transport. To lessen the negative impacts of these conditions, we brought the soils to near saturation with water. By doing so we hoped to facilitate electron transfer via soluble redox mediators and bacterial access to available soluble nutrients in the soils. In addition, we postulated that the near saturated soil conditions would favor biofilm formation and, once formed, these biofilms would exhibit a redox gradient that was increasingly negative from the outside in (Bishop and Yu 1999).

As the cells were not flow through and there was no active transport of carbon or nutrients to the anode electrode surface, we assumed that only the soil microbes in proximity to the anode electrode would be enriched to form an anode biofilm. By filling the anode chamber with sieved and homogenized soil mixtures, impacts from natural soil heterogeneity in terms of microbial diversity were reduced. By collecting the loosely attached anode soil in addition to the direct anode biofilm, we intended to recover both the enriched and the enriching bacterial populations. With regards to the cathodic chamber, no salt was added to the catholyte solution, which is typically done to facilitate electron reduction at the cathode. This choice was made to minimize the likelihood of selective enrichment of halophilic organisms. Although the construction and imposed experimental conditions of each soil cell were non-ideal in terms of power production, the potential for electricity was not fully impeded and obtaining an electrical current was crucial to establishing that the enriched electrode biofilms were at least partially electrogenic in nature.

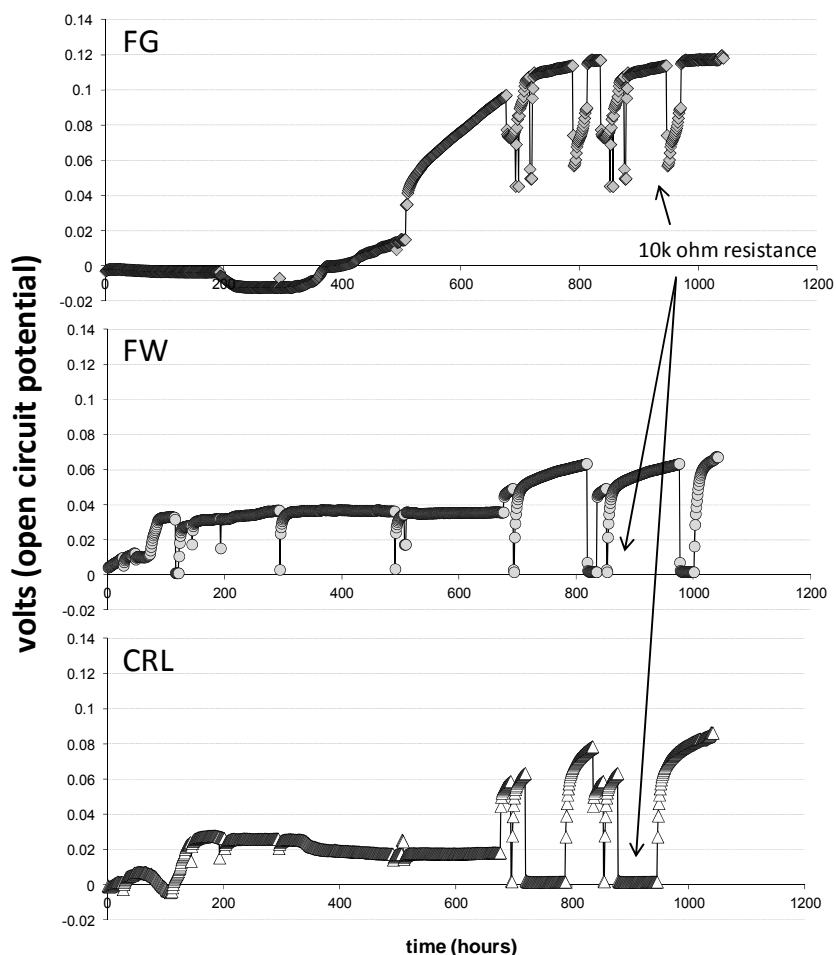


Figure 2. Voltage, open circuit potential, measured from the three soil cells over a 100 hour period. The sharp voltage declines indicate points in time where the output was measured across a 10-k Ω resistor

Results showed all three fuel cells produced electricity. Open circuit potentials (OCP) of between 60 and 120 mV were recorded following an incubation period of 42 days (Fig. 2). Although these voltages were 6 to 110 times less than those recorded from a more efficient soil seeded MFC (Jiang et al. 2010), the increasing and sustained voltages suggested successful bacterial colonization of the embedded graphite anodes. Placing a small load, 10,000 Ω , onto each soil cell had a large effect on the voltage output from each cell, with only the FG cell exhibiting a measurable current of approximately 5 μ A. This soil differed substantially from the other two soils in terms of total organic carbon content, which was five times greater, and iron and manganese content, which were five to eight times greater, respectively (Table 1). In addition, the pH of the FG soil, at 5.6, was substantially lower than that of either the CRL, 6.5, or FW soil, 7.8. Biffinger et al. (2008) examined the effect of pH on an aerobic miniature MFC and found

that pH not only affected biofilm biomass but, more importantly, the production of a soluble redox mediator, riboflavin. In this study, anode microbial biomass recovered from the FG electrode was greater than that recovered from the other two cells, 2.4 nmol FA versus 0.9 to 1.4 nmol (Table 2), and this may explain the 1.5- to 2-fold greater OCP observed with the FG cell. However, pH and specific metabolic functions, discussed below, could equally have influenced biofilm development and influenced electron transport processes. The acquired data only indicate that there was successful colonization of the electrodes in all three soils and that viable mechanisms must have existed for active transfer of electrons to and from the electrodes. Although the mechanisms of electron transfer were not specifically investigated, the bacterial taxonomy of the enriched electrode biofilms was examined in detail.

As indicated above, the recovery of fatty acids from the electrode surfaces of all three soil cells indicated presence of viable microbial biofilms. Fatty acid profiles can provide insight into microbial community abundance and composition and serve as a quantitative measure of community change over time or due to perturbation (Vestal and White 1989). As inocula, all three soils contained a substantial microbial biomass, with little differences being observed top to bottom (Table 2). For the CRL and FW soil cells, the amount of fatty acid recovered from the soils was comparable, averaging 25 to 33 nmol g⁻¹ of soil, respectively, which was slightly greater than that observed in the FG soil, 19 nmol g⁻¹. Assuming 1 pmol of fatty acid is equivalent to 2.5×10^4 bacterial cells (Balkwill et al. 1988), we find that then the cell density for the three soils varied from 5 to 8×10^8 cells per gram of soil.

3.1 Anode biofilms

Although eukaryotic biomarkers were detected on the anode surfaces in all three test cells, this biomass composed less than 10% of the total microbial biomass recovered (Table 2). This result suggests that electron transfer at the anodes was mediated principally by prokaryotes. We assumed that any prokaryotic biomass that was successful in colonizing an anode surface would show a different composition than that present in the surround soil matrix owing to the stark differences in available substrates. Principal components analysis of the prokaryotic fatty acid profiles indicated that differences existed between the compositions of soil and anode bacterial communities (Fig. 3). Factor variable-correlations for the 1st principal component identified the shift or difference in community composition,

soil to anode, to be characterized by increased relative percentages of monounsaturated and normal saturated fatty acids (Table 3). A means comparison of specific prokaryotic FA biomarkers indicated that the normal saturated and monounsaturated FA were significantly different ($p < 0.05$), with the anode biofilms showing the greater relative molar percentages (Fig. 4).

Table 2. Relative molar percentages and absolute abundances of prokaryotic and eukaryotic fatty acid (FA) biomarkers detected in the soils, top middle and bottom fractions, on the electrodes, biofilm and attached soil, and in the catholyte of the three test cells are provided. Biomarker abbreviations are described in the accompanying key.

	CRL soil cell										FW soil cell										FG soil cell									
	anodic chamber					cathodic chamber					anodic chamber					cathodic chamber					anodic chamber					cathodic chamber				
	soils			electrode		soils			electrode		soils			electrode		soils			electrode		soils			electrode		soils			electrode	
	top	middle	bottom	biofilm	attached soil	catholyte	biofilm	top	middle	bottom	biofilm	attached soil	catholyte	biofilm	top	middle	bottom	biofilm	attached soil	catholyte	biofilm	top	middle	bottom	biofilm	attached soil	catholyte	biofilm		
Prokaryote FA biomarkers																														
scnSat %	20	21	19		53	41	25	19	19	18	63	65		25	11	11	11	53	18		18	14								
anteiso-odd %	6	5	5		4	2	1	6	5	5	3	6		1	2	2	2	2	3		3	4								
iso-odd & even %	12	11	11		4	2	1	7	6	7	2	2		2	3	4	4	6	5		6	3								
Cyclo %	3	3	3		4	8	2	3	2	2	4	1		7	1	1	2	1	3		14	6								
Mono %	13	14	13		26	36	24	11	11	11	17	17		38	5	6	5	32	15		39	46								
BrMono %	4	4	3		2	7	3	3	3	3	2	1		5	1	1	1	2	3		6	5								
MidBrSat %	5	5	4		1	0	4	3	3	3	0	0		4	0	1	1	0	1		1	0								
Hopene %	1	1	2		0	0	0	2	2	1	0	0		6	0	0	1	0	1		2	3								
dimeAcetal %	0	0	0		0	0	0	0	0	0	0	0		0	0	0	0	0	0		0	0								
β-hydroxy %	1	1	1		1	0	0	1	1	1	0	1		1	1	1	1	0	1		2	0								
Total prokaryotic %	64	66	62		94	96	60	54	52	50	91	93		89	24	27	27	98	50		90	83								
soil biomass (nmol g-1)	15.53	16.21	16.32					14.11	21.93	14.81					3.63	3.83	7.43													
electrode biomass (nmol)					0.88		2.08				1.28	0.42		4.11				2.33	16.54											
catholyte biomass (nmol ml-1)						1.23															11.73									
Eukaryote FA biomarkers																														
Poly %	2	2	2		2	1	2	1	2	1	2	2		5	0	0	0	1	1		4	2								
dioic %	26	25	27		2	0	37	36	33	38	2	1		0	75	72	70	1	41		1	12								
sterol %	3	3	4		0	0	0	5	8	5	4	0		0	0	0	0	0	1		0	0								
meoxy %	1	1	1		1	3	0	1	1	1	0	0		3	0	0	1	0	1		4	3								
alcohol %	2	2	2		1	1	1	2	3	3	1	5		1	0	0	1	1	5		1	1								
Total eukaryotic %	34	32	36		6	4	40	45	47	49	9	7		10	76	73	72	2	49		9	17								
soil biomass (nmol g-1)	8.18	7.97	9.51					11.89	19.51	14.38					11.56	10.43	19.57													
electrode biomass (nmol)					0.05		1.40				0.13	0.03		0.47				0.05	16.34											
catholyte biomass (nmol ml-1)						0.05															1.19									
total microbial soil biomass (nmol g-1)	24.11	24.54	26.22					26.30	41.95	29.59					15.24	14.35	27.21													
total microbial electrode biomass (nmol)					0.93		3.47				1.41	0.46		4.60				2.38	33.06											
total microbial catholyte biomass (nmol ml-1)						1.28															13.10									
¹ values represent the relative molar percentage each biomarker contributes to overall sample profile.																														
Key:																														
abbrv.	description														functional group															
scnSat	short chain normal saturated (n12:0 - n18:0)														membrane fluidity (prokaryote)															
TerBrSat	terminally branched saturated														Gram-positive bacteria															
iso-odd	iso-odd branched saturated														α-ketoisocaproate pool															
iso-even	iso-even branched saturated														α-ketoisovalerate pool															
anteiso-odd	anteiso-odd branched saturates														α-keto-β-methylvalerate pool															
Mono	monounsaturated														Gram-negative bacteria															
Cyclo	cyclopropyl														CFA synthase (degree of anaerobicity)															
BrMono	methyl branched monounsaturated														dissimilatory sulfate reducer/actinomycete															
MidBrSat	mid-chain methyl branched saturated														actinomycete															
Hopene	hopanoids														many bacteria (diazotrophs); membrane permeability															
dimeAcetal	dimethyl acetals														Gram-positive bacteria; plasmalogen/vinyl ether derived															
β-hydroxy	β-hydroxy saturated (n10:0 - n18:0)														Gram-negative bacteria; component of lipopolysaccharides															
Poly	polyunsaturated														micro-eukaryotes (fungi/protists)															
dioic	di-carboxylated														plant cuticles/some yeasts (lipid oxidative degradation)															
sterol	sterols & stanols														eukaryotes (plants/fungi/protists)															
meoxy	methoxy and oxo fatty acids														algae, fungi and bacteria (mycobacteria); mycolic acids															
alcohol	fatty alcohols														transient lipids; components of wax esters & glycerolipids															

A broad diversity of bacteria has previously been identified in anode biofilms. Proteobacteria, including members of the alpha, beta, gamma, and delta classes, have all been identified as anodophilic organisms when enriched from a variety of inocula that include wastewaters, sewage sludge, and sediments, (Kim et al. 2004; Phung et al. 2004; Kim et al. 2007; Chung and Okabe 2009). Molecular analysis of 16S rRNA genes have resulted in the identification of Firmicutes (typically Gram-positive) and Actinobacteria (often Gram-variable) on the anodes of fuel cells inoculated with wastewaters (Chung and Okabe 2009; Phung et al. 2004, respectively). Results of the fatty acid analyses performed in this study largely agree

with these findings in terms of the types of bacteria detected and expand the data to delineate the community of bacteria present in the adjacent soil from that directly composing the anode biofilms.

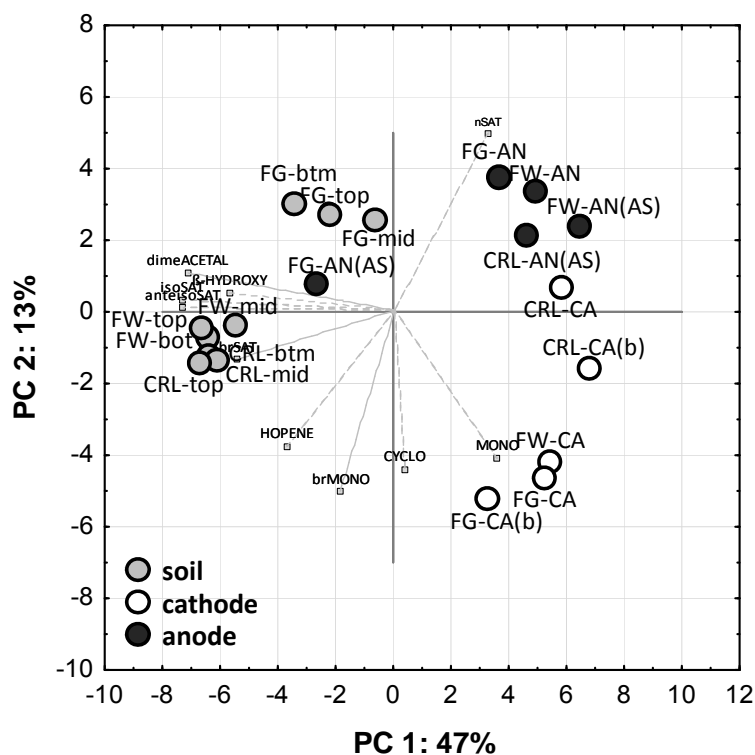


Figure 3. 2D plot illustrating results from the extraction of the 1st two principal components from a dataset comprised of arcsine square root transformed fatty acid molar percentages. Fatty acid profiles recovered from the bulk soil, top, bottom (btm), and middle (mid) fractions, the cathodes, catholyte (b) and cathode biofilm (CA) samples, and the anodes, attached soil (AS) and anode biofilms (AN) for each of the three soil cells, FG, FW, and CRL. The superimposed bi-plot illustrates factor variable correlations (loadings) associated with the first two principal components. The fatty acid functional group abbreviations are described in Table 2.

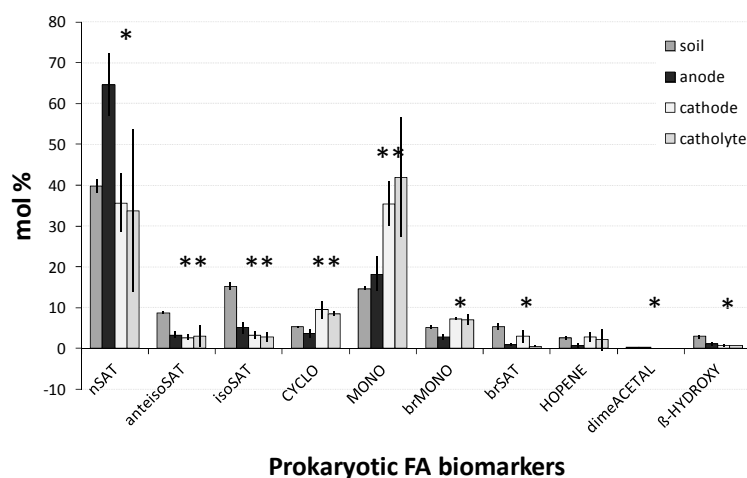


Figure 4. A means comparison of fatty acid methyl ester (FAME) structural class molar percentages (mol%) across the averaged soil profiles (top, middle and bottom, n=9), anode attached soils and biofilms (n=5), cathode biofilms (n=3), and catholyte (n=2). A Tukey HSD post hoc comparison of means identified means that differed significantly at $p < 0.05$ (*) or $p < 0.01$ (**).

Table 3. Factor variable correlations (loadings) between prokaryotic fatty acid (FA) profiles and the 1st principal component that resulted following the principal components analysis of soil, electrode, and catholyte arcsine square root transformed fatty acid molar percentages.

	PC1
Prokaryotic FA biomarkers	factor-variable correlations
monounsaturate	6.20
normal saturate	5.42
cyclopropyl	0.54
CH ₄ branched mnounsaturate	-3.13
mid-chain CH ₄ branched saturate	-8.58
hopene	-10.66
β-hydroxy saturate & unsaturate	-11.84
iso CH ₄ branched saturate	-13.91
anteiso CH ₄ branched saturate	-15.00

Examination of the 16S rRNA gene through terminal restriction fragment length polymorphisms (TRFLP) provided a greater resolution from which anode community similarities and dissimilarities could be determined (Fig. 5). In principle, each terminal restriction fragment (TRF) generated

from a given restriction digest should represent a unique species of bacteria (Marsh 1999). Although this assumption does not always hold true (Blackwood et al. 2007), TRFLP profiles have been successfully used to fingerprint a targeted community and to make comparisons between communities (for example, Kuehl et al. 2005). With the assumption that each identified TRF represents a unique organism, phylotype richness can be estimated from the number of TRF detected. Results from the anode biofilms indicated a similar richness across the three soil cells, with the CRL anode biofilm showing a slightly greater richness, 58 total detected TRF, compared to both the FG and FW cell anodes, 44 and 40 total TRF, respectively. A direct comparison of anode biofilm TRF profiles for the three soil cells indicated both commonalities and differences. Specific TRF, for example Msp I 476 bp, were detected in all three biofilms, suggested the enrichment of a common organism from all three of the soil inocula. Other TRF, for example Rsa I 220 bp, were enriched from a single soil cell. Results indicate that the bacterial taxonomy of the three anode biofilms differed as a result of the starting inocula, but also that the enriched biofilms shared some common characteristics.

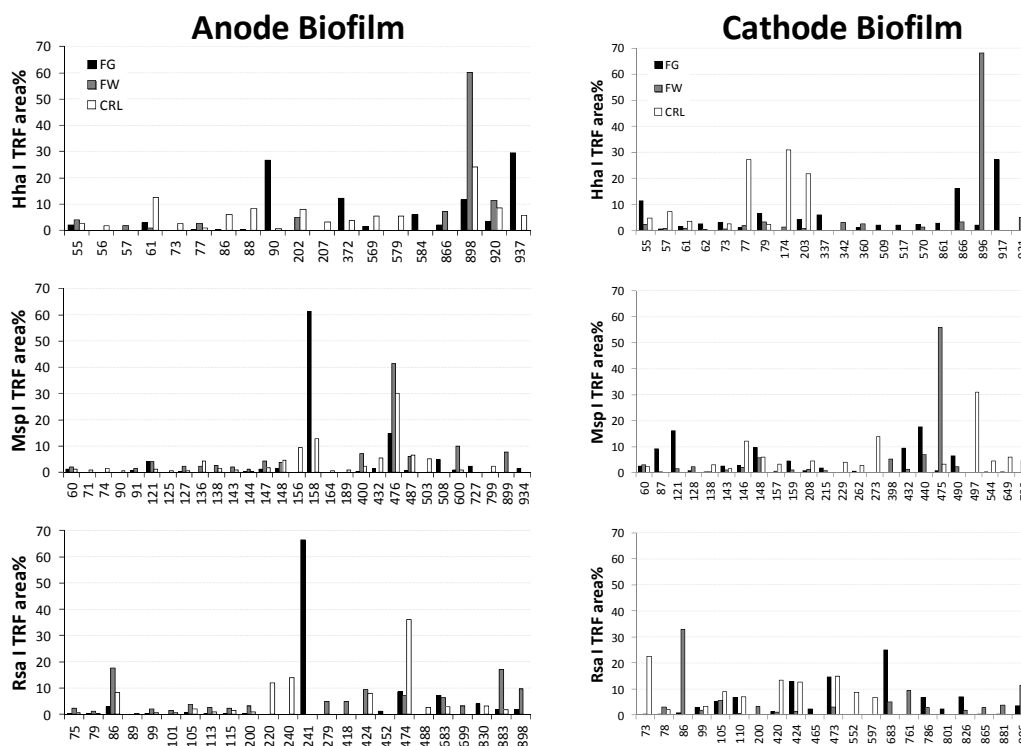


Figure 5. Recovered and retained terminal restriction fragments (TRF) for each of the three soil cells (FG, FW, and CRL) following three restriction digests (Hha I, Msp I, and Rsa I) of bacterial 16S rRNA amplicons of electrode (anode and cathode) biofilm DNA. Each TRF is identified by size as base pairs (bp) and by relative area percentage (area%).

In silico digests of the ribosomal database were then performed to assign putative identifications to the recovered TRF (Table 4). Of the identified genera, the N₂-fixing Rhizobiales were well represented among all three anode biofilms (Fig. 6). The presence of diazotrophs on anode surfaces has also been reported by others. Ishii et al. (2008a) identified a filamentous anode biofilm community composed of a Rhizobiales that developed from rice paddy soil as an inoculum. A *Mesorhizobium* sp. was observed, in situ, by Sukkasem et al. (2008) when examining the anode biofilm of a single chamber air cathode MFC inoculated with wastewater. Saito et al. (2010) recently observed that anodes with an N/C ratio of 0.7 achieved a greater power density than those with an N/C ratio of 3.8, suggesting that the presence of excess nitrogen, which is inhibitory to bacterial nitrogen fixation, limited the growth or activity of diazotrophs on the anode surface. In this study, the soil cell producing the greatest voltage, FG, also showed the lowest N/C ratio, ~10 times lower (Table 1). In addition to the Rhizobiales, all three biofilms showed evidence for the presence of species of burkholderiales, and rhodobacterales. Studies have identified β -proteobacteria, if not Burkholderia sp. specifically, and other α -Proteobacteria such as the Rhodobacterales as members of anode biofilm communities (Phung et al. 2004; Chung and Okabe 2009; Lee et al. 2010). Results from this study indicate that anode colonization by diazotrophic organisms had occurred and that these organisms, Rhizobiales and/or Rhodobacterales likely played an electrogenic role in each of the three soil cells. However, these were not the only organisms putatively identified and, in agreement with the fatty acid analysis, the detection of Firmicutes, Cyanobacteria and δ -Proteobacteria suggests a complexity to the anode bacterial communities.

Both the CRL and FW soil cell anodes yielded putative TRF bacterial identifications that were unique to the particular test cells. Unique to the FW cell anodes was the putative identification of cyanobacteria. Pisciotta et al. (2010) recently demonstrated a light-dependent electrogenic activity in variety of cyanobacteria that appeared to be fundamentally different from the known electrogenic activity of bacteria. Unique to the CRL cell anode was the putative identification of myxococcales. The δ -proteobacteria have been identified on the anodes of a number of MFC systems, and in particular those operated with wastewaters and under anaerobic conditions. Even though the soil cells in this study were not operated anaerobically, the addition of water to near saturation would have favored the formation of anaerobic niches, such as those that occur at surface-biofilm interface (Bishop and Yu 1999). Although differences in soil chemistry likely affected the

development of the anode biofilm communities, it is believed that the community composition is also a reflection of the electrogenic nature of the biofilm (Wrighton et al. 2010).

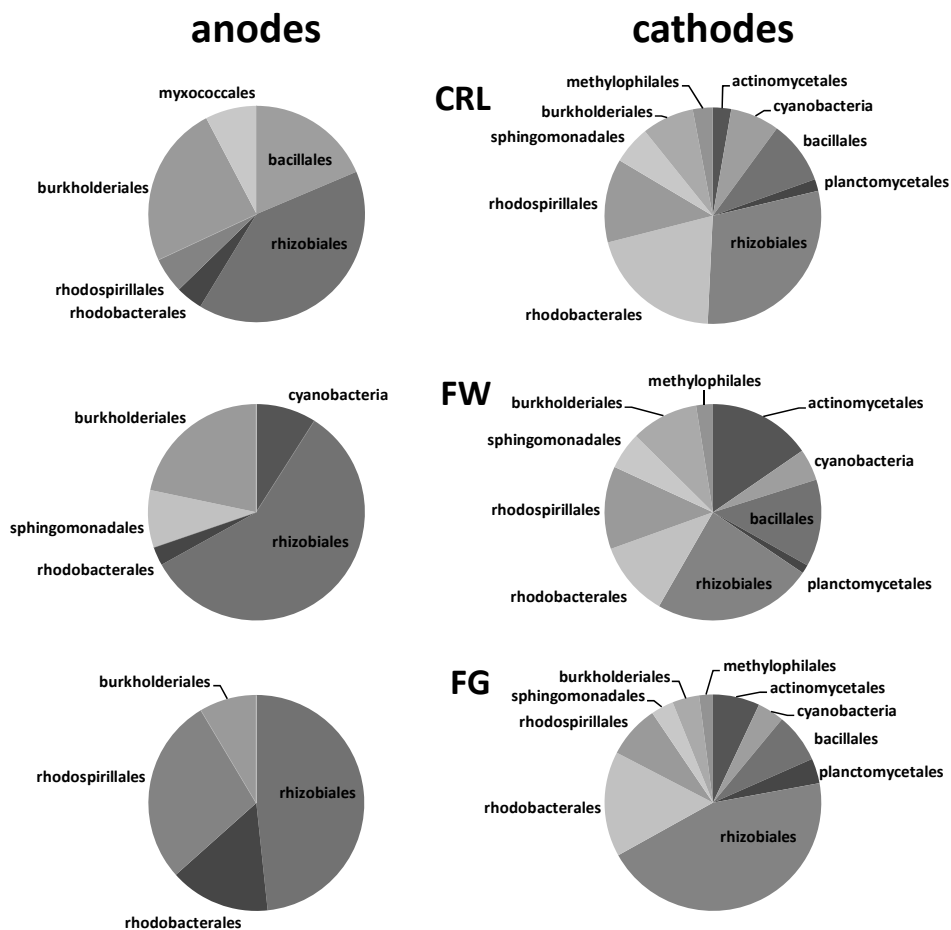


Figure 6. Pie charts of each of the three soil cell anode and cathode biofilm terminal restriction fragments that could be putatively identified through an in silico digest of the ribosomal database. The identified organisms and associated area percentages are provided in Table 4.

Table 4. Putative identifications for retained terminal restriction fragments of amplified 16S rRNA DNA recovered from the anodes and cathodes of the three test cells following the in silico digest of the ribosomal database. Sample TRF that could be matched to an organism are provided along with the normalized (to 100%) relative area percentages.

phylum	class	order	genera	TRF (bp)			anodes normalized area % ¹			cathodes normalized area %		
				Hha I	Msp I	Rsa I	CRL	FG	FW	CRL	FG	FW
Actinobacteria	Actinobacteria	Actinomycetales	<i>Kribbella</i> sp.	174	143	78				0.9	1.3	4.7
				159								
			<i>Mycobacterium</i> sp.	174	128	78				0.8	2.3	5.0
				360	157							
				159								
			<i>Rhodococcus</i> sp.	174	138	78				0.7	1.8	5.4
				360	157							
				159								
			<i>Streptomonospora</i> sp.	360	128	78				0.3	1.6	0.3
			<i>Cylindrospermopsis</i> sp.	866	148	424			9.0	5.7	3.3	2.9
Firmicute	Bacilli	family 1		60								
				490								
			<i>Prochlorococcus</i> sp.	337	490	420				1.6	0.7	1.9
			<i>Acetobacter</i> sp.	579	147	424	4.6					
				488								
			<i>Bacillus</i> sp.	61	146	473				5.8	5.1	9.3
				79	138	105						
				148	420							
				99								
			<i>Halobacillus</i> sp.	61	148	473				3.6	2.3	3.8
Planctomycetes	Planctomycetacia	Planctomycetales		157								
			<i>Pirellula</i> sp.	61	148	896				1.8	3.8	1.3
			<i>Altipia</i> sp.	342	440	110				4.4	2.5	2.7
				424								
			<i>Bacillus</i> sp.?	61	136	105		12.3	9.3			
				144	474							
				147	492							
				148								
			<i>Blastochloris</i> sp.	61	439	117		3.1				
			<i>Bradyrhizobium</i> sp.	61	152	110	5.8	2.7	7.1			
Proteobacteria	alpha	Rhizobiales		400	424							
			<i>Brucella</i> sp.	61	400	105	4.4	2.8	4.7	0.8	2.4	1.7
			<i>Labrys</i> sp.	61	127	105	4.0	2.8	2.8	3.1	4.1	3.6
				342	440	420						
			<i>Mesorhizobium</i> sp.	61	100	683	4.9	7.7	7.1	3.3	3.7	0.5
				127	761							
				400								
			<i>Methylophilus</i> sp.	61	439	115		3.4				
				117								
			<i>Ochrobactrum</i> sp.	61	400	105	4.4	2.8	4.7	6.4	6.3	4.3
Proteobacteria	beta	Burkholderiales		342	148	424						
				440	826							
			<i>Rhizobium</i> sp.	61	127	105	4.6	3.4	5.6	5.5	20.3	3.5
				342	400	110						
				475	105							
				440	826							
				60	896							
			<i>Rhodoplanes</i> sp.	61	148	105	7.2	3.6	7.2	3.5	2.8	4.3
				424								
			unknown	61	127	105	4.9	3.8	9.5	2.4	2.7	3.4
Proteobacteria	gamma	Rhodobacterales		342	152	110						
				400	117							
			<i>Octadecabacter</i> sp.	61	440	424				3.8	1.9	2.2
			<i>Oleiphilus</i> sp.	61	439	117		3.1				
			<i>Paracoccus</i> sp.	61	438	117		2.3				
			<i>Pelagibaca</i> sp.	61	127	105	4.0	3.6	2.8	3.0	3.3	1.7
				440								
			<i>Phaeobacter</i> sp.	61	440	826				3.1	2.0	0.5
			<i>Roseobacter</i> sp.	61	440	826				3.1	2.6	0.5
				342								
Proteobacteria	delta	Methylophilales	<i>Sulfitobacter</i> sp.	61	439	117		3.1		3.9	2.7	4.1
				342	420							
				424								
			unknown	61	439	117		3.1		3.3	3.3	2.3
				342	128	420						
				826								
			<i>Acetobacter</i> sp.	174	146	424				4.0	2.5	7.6
				440								
			<i>Acidiphilium</i> sp.	517	440	424				3.9	1.7	1.7
			<i>Azospirillum</i> sp.	88	157	830	5.3					
Proteobacteria	delta	Myxococcales	unknown	61	439	683		28.1		4.7	3.7	3.0
				90	148	420						
				79	159							
			<i>Sphingomonas</i> sp.	79	100	418			8.6	5.7	3.4	5.6
				147	424							
				440	826							
			<i>Achromobacter</i> sp.	570	490	473				2.8	1.4	2.0
			<i>Burkholderia</i> sp.	79	143	474	10.4		4.4	2.3	1.1	2.3
				207								
				570								
Proteobacteria	delta	Methylophilales	<i>Polaromonas</i> sp.	202	487	424	6.8		8.1			
				207								
			<i>Telluria</i> sp.	77	490	473				2.7	1.5	5.8
				202	138	117	7.1	8.5	9.2			
				207	487	424						
				569	495	474						
			<i>Methylophilus</i> sp.	360	490	99				3.0	2.0	2.5
				473								
			<i>Myxococcus</i> sp.	61	158	488	7.7					
				161								

In addition to the above described in situ analyses, we also performed enrichments and isolation to obtain single organisms for future study. Those bacteria susceptible to enrichment, isolation, and identifications are provided in Table 5. All three anodes yielded Burkholderiales, Rhodobacterales, and Bacillales, all in agreement with the TRFLP results. In addition, a large number of Actinomycetales were isolated from the anode biofilms. Unfortunately, the lack of enrichment of Rhizobiales may reflect a bias attributable to media formulations and/or culture conditions. Nevertheless, the in situ community analyses by FA indicated an enrichment in monounsaturated FA onto anode surfaces, which are representative of Gram-negative bacteria such as the Proteobacteria. The in situ analysis by TRFLP also putatively identified the presence of Proteobacteria on the anode surfaces of all three test cells. Lastly, species of α - and β -Proteobacteria were enriched and isolated from the anode surfaces. Voltage output from the FG soil cell was approximately 2-fold greater than that of either the FW or CRL cells. Under the assumption that the electrogenic nature of the biofilms is reflected by the extant community composition, then the FG anode surface should show a distinguishing characteristic.

The relative percentage of monounsaturated FA from the FG biofilm, 32%, was slightly greater than that extracted from either the FW, 17%, or the CRL, 26%, anode biofilms (Table 2). The relative percentage of FG biofilm TRF putatively identified as being from α -Proteobacteria, 92%, was far greater than those recovered from the FW or CRL biofilms, 69 and 49%, respectively (Table 4). In contrast, TRF putatively identified as belonging to β -Proteobacteria were detected at only 8% in the FG biofilm compared to 22 and 32% in the FW and CRL biofilms. A similar result was also seen with the percentage of TRF putatively identified as belonging to Firmicutes. The results are consistent in identifying the α -Proteobacteria as substantial colonizers of graphite anodes and suggest that their abundance is directly related to the electrogenic capacity of the anodes.

Table 5. Enriched, isolated, and identified bacteria recovered from the anode and cathode biofilms of each of the three test cells. Bacteria were identified by fatty acid profile using the Sherlock identification system.

soil	isolate ID	electrode	Identification	index	phylum	class	order
FG	i17	anode(attached)	Kocuria-kristinae	0.697	Actinobacteria	Actinobacteria	Actinomycetales
	i18	anode(attached)	Bacillus-cereus-GC subgroup A	0.823	Firmicute	Bacilli	Bacillales
	i26	anode(loose)	Kocuria-kristinae	0.724	Actinobacteria	Actinobacteria	Actinomycetales
	i25	anode(loose)	Bacillus-mycoides-GC subgroup A (Bacillus cereus group)	0.651	Firmicute	Bacilli	Bacillales
	i27	anode(loose)	Paenibacillus-polymyxa (Bacillus)	0.746	Firmicute	Bacilli	Bacillales
	i28	anode(loose)	Brevundimonas-vesicularis (Pseudomonas vesicularis)	0.309	Proteobacteria	α -proteobacteria	Caulobacteriales
	i23	cathode	Arthrobacter-globiformis-GC subgroup A (some 48h)	0.565	Actinobacteria	Actinobacteria	Actinomycetales
	i21	cathode	Kocuria-kristinae	0.706	Actinobacteria	Actinobacteria	Actinomycetales
	i22	cathode	Micrococcus-luteus-GC subgroup B (includes ATCC 9341)	0.619	Actinobacteria	Actinobacteria	Actinomycetales
FW	i39	anode(attached)	Arthrobacter-oxydans	0.646	Actinobacteria	Actinobacteria	Actinomycetales
	i35	anode(attached)	Rhodococcus-erythropolis/R. globerulus/N. globerula	0.952	Actinobacteria	Actinobacteria	Actinomycetales
	i36	anode(attached)	Bacillus-cereus-GC subgroup A	0.715	Firmicute	Bacilli	Bacillales
	i31	anode(attached)	Bacillus-megaterium-GC subgroup A	0.908	Firmicute	Bacilli	Bacillales
	i40	anode(attached)	Brevibacillus-parabrevis-GC subgroup B	0.460	Firmicute	Bacilli	Bacillales
	i32	anode(attached)	Paracoccus-denitrificans	0.461	Proteobacteria	α -proteobacteria	Rhodobacteriales
	i52	anode(loose)	Arthrobacter-oxydans	0.847	Actinobacteria	Actinobacteria	Actinomycetales
	i48	anode(loose)	Kocuria-kristinae	0.880	Actinobacteria	Actinobacteria	Actinomycetales
	i45	anode(loose)	Bacillus-megaterium-GC subgroup A	0.803	Firmicute	Bacilli	Bacillales
	i51	anode(loose)	Brevibacillus-parabrevis-GC subgroup B	0.769	Firmicute	Bacilli	Bacillales
	i47	anode(loose)	Janthinobacterium-lividum	0.836	Proteobacteria	β -proteobacteria	Burkholderiales
	i49	anode(loose)	Paracoccus-denitrificans	0.619	Proteobacteria	α -proteobacteria	Rhodobacteriales
	i43	cathode	Microbacterium-flavescens (Aureobacterium, Arthrobacter)	0.059	Actinobacteria	Actinobacteria	Actinomycetales
	i44a,b	cathode	Micrococcus-luteus-GC subgroup A	0.222	Actinobacteria	Actinobacteria	Actinomycetales
	i42	cathode	Stenotrophomonas-maltophilia (Xanthomonas, Pseudomonas)	0.165	Proteobacteria	γ -proteobacteria	Xanthomonadales
CRL	i1	anode(attached)	Bacillus-cereus-GC subgroup A	0.352	Firmicute	Bacilli	Bacillales
	i5	anode(attached)	Ralstonia-eutropha (Alcaligenes eutrophus)	0.946	Proteobacteria	β -proteobacteria	Burkholderiales
	i16	anode(loose)	Bacillus-cereus-GC subgroup A	0.610	Firmicute	Bacilli	Bacillales
	i12	cathode	Arthrobacter-globiformis-GC subgroup A (some 48h)	0.366	Actinobacteria	Actinobacteria	Actinomycetales
	i10	cathode	Kocuria-kristinae	0.684	Actinobacteria	Actinobacteria	Actinomycetales
	i9	cathode	Microbacterium-barkeri (Aureobacterium, Corynebacterium)	0.654	Actinobacteria	Actinobacteria	Actinomycetales

3.2 Cathode Biofilms

Biocathodes facilitate the efficient reduction of oxygen within the cathodic chamber and the composition of cathode biofilms can also reflect the performance of an MFC (Clauwaert et al. 2007). Wrighton et al. (2010) determined that bacterial community structure of cathode biofilms corresponded to MFC performance and that α - and γ -Proteobacteria, and Firmicutes, made up a large percentage of the cathode community. In contrast to this study, the cathode communities described by Wrighton et al. (2010) were initiated from wastewater treatment sludge and propagated in a minimal nutrient media. Considering that no nutrient supplements were added to the cathodic chamber in this study, other than the small aliquot of soil, the presence of any biofilm at all suggests a selective enrichment of bacteria capable of using the cathode, at least in part, as a source of electrons.

Fatty acid profiles and abundances in the catholyte and cathode biofilms were found to be similar, which is attributed to the restrictive nature of the

growth media present in the cathode chamber (Fig. 3 and 4). Cathode biofilms also showed a diversity of prokaryotic FA, which was composed primarily of monounsaturated FA and to lesser extents by terminally branched and mid-chain branched saturated FA, a similar result to that observed with the anode biofilms. The diversity of FA detected on the cathodes suggests some complexity to the composition of these biofilm communities, which was similar in nature to that described for the anode biofilms. However, the two electrode biofilms did differ substantially in the amount of eukaryotic biomass detected, which constituted between 10 and 40% of total cathode biofilm microbial biomass (Table 2). Of the eukaryotic functional groups detected, dioic fatty acids, which are indicative of some yeasts, were prominent in both the CRL and FG soil cells. Yeasts have been shown to mediate electron transfer to MFC anodes and are considered by some to be ideal electrogenic organisms owing to their growth rates and broad spectrum for substrates (Gunawardena et al. 2008; Ganguli and Dunn 2009; Haslett et al. 2011). Neither molecular nor enrichments/isolations were performed to further identify any cathode eukaryotes; however, the presumed presence of higher organisms on the cathodes suggests that these organisms may have played a role in mediating electron transfer from the electrodes.

Bacterial communities on the cathodes were also further evaluated through TRFLP analysis of amplified 16S rRNA gene fragments. Unexpectedly, TRF recovered from the biocathodes indicated a phylotype richness that was 14–20% greater than that observed on the respective anodes in the FG and FW cells, but 45% lower in the CRL cell (Fig. 5). The formation of similar, but not identical, biofilms on both anodes and cathodes was not anticipated. We assumed that the prevalence of carbon substrates present in the soils surrounding the anode electrodes would produce a biofilm considerably different from that forming on the cathodes. The finding of similar communities on both electrodes suggests a specific influence of the graphite surfaces on biofilm formation. Although the catholyte was not augmented with nutrients or carbon substrates other than that present in the initial inoculums, the results of both the FA and TRFLP assays indicate that conditions within the cathodic chamber were sufficient to support the growth of a biofilm as or more complex than that observed on the corresponding soil embedded anodes.

A number of recovered TRF could also be putatively assigned to bacterial species (Table 4). Rhizobiales, Rhodobacterales, Rhodospirillales,

Burkholderiales, and Bacillales were prominent in each of the three cathode biofilms. These are the same orders that were found to be prominent in the anode biofilms. In addition, Actinomycetales were putatively identified in each of three biofilms and these were the only order of bacteria, other than a single γ -Proteobacteria, that were susceptible to enrichment and isolation from the cathode biofilms (Table 5). Of the cultured Actinomycetales, species of *Arthobacter* and *Micrococcus* have been identified as electrogenic and effectively used as anode inocula (Hou et al. 2011; Choi et al. 2007). Although Wrighton et al. (2010) did identify the presence of actinobacteria on a biocathode, these organisms were not found to be predominant, a finding similar to this study. These non-spore forming cocci are both chemoorganotrophic and usually grow on simple media. Their ability to utilize a variety of substrates for metabolism likely enabled their colonization of the soil cell cathodes.

The presence of diazotrophs on both the anodes and cathodes may suggest a dual role for these organisms. As discussed above, Saito et al. (2010) suggested that lower N/C ratios at the anode could translate into greater MFC power densities. We also observed a greater voltage output in a soil cell that exhibited a low N/C ratio. However, N/C ratios at the cathodes should have been similar for all three cells because of the consistency of bulk media type and the minimal amount, 0.1 g, of soil added. As the catholyte was not amended with organic nitrogen, the ability to fix atmospheric nitrogen would have been advantageous to organisms within the cathode biofilm. Nitrogen fixing activity at the cathode, as evidenced by the putative identification of Rhizobiales, could have made a product for subsequent nitrification and denitrification. Although nitrifiers were not specifically identified from the *in silico* digests, potential denitrifying populations were. Similar to the result of Wrighton et al. (2010), members of the Burkholderiales and Bacillales were prominent on the cathodes of all three test cells examined in this study. It is possible that the diversity of organisms putatively identified on the cathodes describes a complex community that supports a coupled nitrogen cycle, one that ultimately facilitates the reduction of oxygen at the electrode surface.

4 Summary and Conclusion

The simple cells proved to be sufficient test vessels for examining the development of anodophilic and cathodophilic soil organisms. The recovery of a microbial biomass from both anodes and cathodes and the identification of community differences between the inocula and the biofilms that formed indicated that a selective enrichment had occurred. The measurement of an electrical output suggested that these enriched biofilms were, at least partially, electrogenic in nature. Polyphasic analysis of anode biofilm taxonomy identified an enrichment of Proteobacteria, specifically α -Proteobacteria. In situ analyses indicated a predominance of Rhizobiales and differences in soil geochemistry, specifically the N/C ratios, suggested a correlation between the abundance of these organisms and the electrogenic capacity of the anode biofilms. In the cathode chamber, the diversity in situ biomarkers detected in the electrode biofilms suggested some complexity to the composition of these biofilm communities that was not only similar in nature to that of the anode biofilms, but also of greater phylotype richness. One distinguishing characteristic of the cathode biofilms was an abundance of an eukaryotic biomass, specifically one attributed to yeasts. Results of this study identified the existence of complex biofilms on both anodes and cathodes. The diversity and types of organisms identified on the electrode surfaces in this study were similar to the findings of others, and as a whole these results suggest that the turnover of nitrogen at the electrode surfaces, anode or cathode, can play a significant role in MFC function.

References

- Alterman, P., K. Rabaey, P. Clauwaert, and W. Verstraete. 2006. Microbial fuel cells for wastewater treatment. *Wat. Sci. Technol.* **54**(8): 9–15.
- Balkwill D. L, F. R. Leach, J. T. Wilson, J. F. McNabb, and D. C. White. 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface sediments. *Microb Ecol.* **16**: 73–84.
- Biffinger, J. C., J. Pietron, O. Bretschger, L. J. Nadeau, G. R. Johnson, C. C. Willimas, K. H. Neilson, and B. R. Ringeisen. 2008. The influence of acidity on microbial fuel cells containing *Shewanella oneidensis*. *Biosens. Bioelectron.* **24**(4): 906–911.
- Bishop, P. L., and T. Yu. 1999. A microelectrode study of redox potential change in biofilms. *Wat. Sci. Technol.* **39**(7): 179–185.
- Blackwood, C. B., D. Hudleston, D. R. Zak, and J. S. Buyer. 2007. Interpreting ecological diversity indices applied to terminal restriction fragment length polymorphism data: Insights from simulated microbial communities. *Appl. Environ. Microbiol.* **73**(16): 5276–5283.
- Chaudhuri, S. K., and D. R. Lovely. 2003. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. *Nature Biotech.* **21**(10): 1229–1232.
- Choi, Y., E. Jung, H. Park, S. Jung, and S. Kim. 2007. Effect of initial carbon sources on the performance of a microbial fuel cell containing environmental microorganism *Micrococcus luteus*. *Notes* **28**: 1591.
- Chung, K., and S. Okabe. 2009. Continuous power generation and microbial community structure of the anode biofilms in a three-stage microbial fuel cell system. *Appl. Microbiol. Biotechnol.* **83**: 965–977.
- Clauwaert, P., D. Van Der Ha, N. Boon, K. Verbeken, M. Verhaege, K. Rabaey, and W. Verstraete. 2007. Open air biocathode enables effective electricity generation with microbial fuel cells. *Environ. Sci. Technol.*
- Dunbar, J., L. O. Ticknor, and C. R. Kuske. 2000. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl. Environ. Microbiol.* **67**(1): 190–197.
- Ganguli, R., and B. S. Dunn. 2009. Kinetics of anode reactions for a yeast-catalysed microbial fuel cell. *Fuel Cells* **9**: 44–52.
- Gunawardena, A., S. Fernando, and F. To. 2008. Performance of a yeast-mediated biological fuel cell. *Int. J. Mol. Sci.* **9**(10): 1893–1907.
- Haslett, N. D., F. J. Rawson, F. Barriere, G. Kunze, N. Pasco, R. Gooneratne, and K. H. Baronian. 2011. Characterization of yeast microbial fuel cell with the yeast *Arxula adenivorans* as the biocatalyst. *Biosens. Bioelectron.* Epub ahead of print.

- Hou, H., L. Li, P. de Figueiredo, and A. Han. 2011. Air-cathode microbial fuel cell array: a device for identifying and characterizing electrochemically active microbes. *Biosens. Bioelectron.* **26**(5): 2680–2684.
- Ishii, S., T. Shimoyama, Y. Hotta, and K. Watanabe. 2008a. Characterization of a filamentous biofilm community established in a cellulose-fed microbial fuel cell. *BMC Microbiol* **8**: 6.
- Ishii, S., Y. Hotta, and K. Watanabe. 2008b. Methanogenesis versus electrogenesis: morphological and phylogenetic comparisons of microbial communities. *Biosci Biotechnol. Biochem.* **72**(2): 286–294.
- Jiang, D., B. Li, W. Jia, and Y. Lei. 2010. Effect of inoculum types on bacterial adhesion and power production in microbial fuel cells. *Appl. Biochem. Biotechnol.* **160**: 182–196.
- Kim, B. H., H. S. Park, H. J. Kim, G. T. Kim, I. S. Chang, J. Lee, and N. T. Phung. 2004. Enrichment of microbial community generating electricity using a fuel-cell-type electrochemical cell. *Appl. Microbiol. Biotechnol.* **63**: 672–681.
- Kim, J.R., S. H. Jung, J. M. Regan, and B. E. Logan. 2007. Electricity generation and microbial community analysis of alcohol powered microbial fuel cells. *Bioresour. Tech.* **98**: 2568–2577.
- Kroppenstedt, R. M. 1985. Fatty acid and menaquinone analysis of actinomycetes and related organisms. In: *Chemical Methods in Bacterial Systematics* (M. Goodfellow and D. E. Minnikin, Eds). SAB Technical Series 20, Academic Press, London, pp. 173–199.
- Kuehl, C. J., H. D. Wood, T. L. Marsh, T. M. Schmidt, and V. B. Young. 2005. Colonization of the cecal mucosa by *Helicobacter hepaticus* impacts the diversity of the indigenous microbiota. *Infect. Immun.* **73**(10): 6952–6961.
- Lee, T. K., T. V. Doan, K. Yoo, S. Choi, C. Kim, and J. Park. 2010. Discovery of commonly existing anode biofilm microbes in two different wastewater treatment MFCs using FLX Titanium pyrosequencing. *Appl Microbiol. Biotechnol.* **87**: 2335–2343.
- Liu, H., and B. E. Logan. 2004. Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. *Environ. Sci. Technol.* **38**: 4040–4046.
- Liu, W., A. Wang, S. Cheng, B. E. Logan, H. Yu, Y. Deng, J. D. Van Nostrand, L. Wu, Z. He, and J. Zhou. 2010. Geochip-based functional gene analysis of anodophilic communities in microbial electrolysis cells under different operational modes. *Environ. Sci. Technol.* **44**: 7729–7735.
- Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): An emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* **2**: 323–327.
- O’Leary, W. M., and S. G. Wilkinson. 1988. Gram-positive bacteria. In: *Microbial Lipids* (C. Ratledge and S. G. Wilkinson Eds) Volume 1. Academic Press, p. 117–185.

- Oliveros, J. C. 2007. *VENNY. An interactive tool for comparing lists with Venn Diagrams*. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>.
- Pham, T. H., N. Boon, P. Aelterman, P. Clauwaert, L. D. Schampelaire, L. Vanhaecke, K. D. Maeyer, M. Höfte, W. Verstraete, and K. Rabaey. 2008. Metabolites produced by *Pseudomonas* sp. enable a Gram-positive bacterium to achieve extracellular electron transfer. *Appl. Microbiol. Biotechnol.* **77**: 1119–1129.
- Phung, N. T., J. Lee, K. H. Kang, I. S. Chang, G. M. Gadd, and B. H. Kim. 2004. Analysis of microbial diversity in oligotrophic microbial fuel cells using 16S rDNA sequences. *FEMS Microbiol. Lett.* **233**: 77–82.
- Pisciotta, J. M., Y. Zou, and I. V. Baskakov. 2010. Light-dependent electrogenic activity of cyanobacteria. *PLoS ONE* **5**(5): e10821.
- Potter, M. C. 1912. Electrical effects accompanying the decomposition of organic compounds. *Proc Roy Soc (London)* **84**(B): 260–276.
- Rabaey, K., N. Boon, S. D. Siciliano, M. Verhaege, and W. Verstraete. 2004. Biofuel cells select for microbial consortia that self-mediate electron transfer. *Appl Environ. Microbiol.* **70**(9): 5373–5382.
- Rabaey, K., N. Boon, M. Hofte, and W. Verstraete. 2005. Microbial phenazine production enhances electron transfer in biofuel cells. *Environ. Sci. Technol.* **39**: 3401–3408.
- Reguera, G., K. D. McCarthy, T. Mehta, J.S. Nicoll, M. T. Tuominen, and D. R. Lovely. 2005. Extracellular electron transfer via microbial nanowires. *Nature*. **435**: 1098–1101.
- Ringeisen, B. R., E. Henderson, P. K. Wu, J. Pietron, R. Ray, B. Little, J. C. Biffinger, and J.M. Jones-Meehan. 2006. High power density from a miniature microbial fuel cell using *Shewanella oneidensis* DSP10. *Environ. Sci. Technol.* **40**: 2629–2634.
- Saito, T., M. Mehanna, X. Wang, R. D. Cusick, Y. Feng, M. A. Hickner, and B. E. Logan. 2010. Effect of nitrogen addition on the performance of microbial fuel cell anodes. *Bioresour. Tech.* **102**: 395–398.
- Schampelaire, L. D., A. Cabezas, M. Marzorati, M. W. Friedrich, N. Boon, and W. Verstraete. 2010. Microbial community analysis of anodes from sediment microbial fuel cells powered by rhizodeposits of living rice plants. *Appl Environ. Microbiol.* **76**(6): 2002–2008.
- Schampelaire, L. D., L. Van Den Bossche, H.S. Dang, M. Hofte, N. Boon, K. Rabaey, and W. Verstraete. 2008. Microbial fuel cells generating electricity from rhizodeposits of rice plants. *Environ. Sci. Technol.* **42**: 3053–3058.
- Shantaram, A., H. Beyenal, R. Raajan, A. Veluchamy, and Z. Lewandowski. 2005. Wireless sensors powered by microbial fuel cells. *Environ. Sci. Technol.* **39**: 5037–5042.
- Shyu, C., T. Soule, S. J. Bent, J. A. Foster, and L. J. Forney. 2007. MiCA: A web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA genes. *J. Microb. Ecol.* **53**: 562–570.

- Sukkasema, C., S. Xua, S. Parka, B. Boonsawang, and H. L. Sukkasem. 2008. Effect of nitrate on the performance of single chamber air cathode microbial fuel cells. *Wat. Res.* **42**(19): 4743–4750.
- Vestal, J. R., and D. C. White. 1989. Lipid analysis in microbial ecology: Quantitative approaches to the study of microbial communities. *BioScience*, **39**(8): 535–541.
- White, D. C., and D. B. Ringelberg. 1998. Signature lipid biomarker analysis. In: *Techniques in Microbial Ecology* (R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Saylor Eds). New York: Oxford University Press, Inc., p. 255–272.
- Wrighton, K. C., B. Virdis, P. Clauwaert, S. T. Read, R. A. Daly, N. Boon, Y. Piceno, G. L. Andersen, J.D. Coates, and K. Rabaey. 2010. Bacterial community structure corresponds to performance during cathodic nitrate reduction. *ISME J.* **4**: 1443–1455.
- Wilkinson, S. G. 1988. Gram-negative bacteria, In: *Microbial Lipids* (C. Ratledge and S. G. Wilkinson Eds) Volume 1. Academic Press, p. 299–457.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) April 2012		2. REPORT TYPE		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE Community Composition of Bacterial Biofilms Formed on Simple Soil Based Bioelectrochemical Cell Anodes and Cathodes				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) David B. Ringelberg, Karen L. Foley, and Charles M. Reynolds				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cold Regions Research and Engineering Laboratory U.S. Army Engineer Research and Development Center 72 Lyme Road Hanover, NH 03755				8. PERFORMING ORGANIZATION REPORT NUMBER ERDC/CRREL TR-12-2	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Installation Technology Program U.S. Army Corps of Engineers				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Microbial fuel cells (MFC), as bioelectrochemical systems, hold promise as a sustainable source of energy for use in novel environments and settings. Although electrode biofilms, both anode and cathode, are critical to the production of power in these systems, the taxonomies of the biofilms that form are not fully understood. The specific objectives of the current study were to classify the bacteria that enriched onto fuel cell electrodes, with three biogeochemically distinct surface soils serving as the inocula. Following 1000 hours of incubation under saturated soil conditions, the community composition of the anode and cathode bacterial biofilms was quantified by culture, fatty acid profile (FA), and terminal restriction fragment length polymorphisms (TRFLP). The three soils produced open circuit voltages of between 60 and 120 mV, but only one soil maintained voltage under resistance (60 mV at 10,000 Ω). Fatty acid profiling identified differences among anode, cathode, and bulk soil microbial communities, specifically a greater relative abundance of terminally branched saturated FA in the bulk soils and a greater relative abundance of monounsaturated and cyclopropane FA on electrode surfaces. Bacteria cultured from both the anode and cathode biofilms included species of Actinobacteria; however, only the anode biofilms produced species of Firmicutes and Proteobacteria. Analysis of TRFLP profiles putatively identified a diversity of bacteria in the biofilms recovered from both electrode surfaces. The most predominant organisms detected were the α - and β -Proteobacteria, specifically the Rhizobiales, Rhodobacterales, and Burkholderiales. Although the detection of similar community fingerprints on both anodes and cathodes was not anticipated, the diversity of organisms putatively identified indicated a complexity that would support a coupled nitrogen cycle, one capable of facilitating the transfer of electrons to the soil cell anodes and from the soil cell cathodes.					
15. SUBJECT TERMS Bioelectrochemical cell Fatty acid profile				Microbial community composition TRFLP	
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (include area code)
U	U	U	none	35	